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RESEARCH ARTICLE

MeCP2 dysfunction prevents proper BMP signaling and neural progenitor expansion in brain organoid

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Abstract

Objectives: Sporadic mutations in MeCP2 are a hallmark of Rett syndrome (RTT). Many RTT brain organoid models have exhibited pathogenic phenotypes such as decreased spine density and small size of soma with altered electrophysiological signals. However, previous models are mainly focused on the phenotypes observed in the late phase and rarely provide clues for the defect of neural progenitors which generate different types of neurons and glial cells. **Methods**: We newly established the RTT brain organoid model derived from MeCP2-truncated iPS cells which were genetically engineered by CRISPR/Cas9 technology. By immunofluorescence imaging, we studied the development of NPC pool and its fate specification into glutamatergic neurons or astrocytes in RTT organoids. By total RNA sequencing, we investigated which signaling pathways were altered during the early brain development in RTT organoids. **Results**: Dysfunction of MeCP2 caused the defect of neural rosette formation in the early phase of cortical development. In total transcriptome analysis, BMP pathway-related genes are highly associated with MeCP2 depletion. Moreover, levels of pSMAD1/5 and BMP target genes are excessively increased, and treatment of BMP inhibitors partially rescues the cell cycle progression of neural progenitors. Subsequently, MeCP2 dysfunction reduced the glutamatergic neurogenesis and induced overproduction of astrocytes. Nevertheless, early inhibition of BMP pathway rescued *VGLUT1* expression and suppressed astrocyte maturation. **Interpretation**: Our results demonstrate that MeCP2 is required for the expansion of neural progenitor cells by modulating BMP pathway at early stages of development, and this influence persists during neurogenesis and gliogenesis at later stages of brain organoid development.

Introduction

Rett syndrome (RTT) is a neurodevelopmental disorder which exhibits the gradual regression of brain function at 12–18 months after birth, including impairment of cognitive and motor development, ataxia, seizure, and autistic-like behaviors.[1–4] Almost all RTT patients have loss-of-function mutations of methyl-CpG binding protein 2 (MeCP2), a transcriptional regulator required for brain development and normal function.[1,5,6] Dysfunction of MeCP2 causes the impairment of neuronal differentiation, maturation, and neural circuit formation, which lead to a broad range of neurological symptoms in RTT patients.[1,7,8]

In recent decades, 3D brain organoids have emerged as a new strategy for modeling of various brain disorders because they highly recapitulate brain development in diverse cell types and exhibit key features of target diseases.[9–11] In particular, patient-derived induced pluripotent stem cells (iPSCs) with so-called hotspot mutations of MeCP2 are commonly used for the RTT brain organoid model presenting abnormal neurogenesis.[12–14] MeCP2 R255X mutant cortical organoids showed

premature formation of the deep-cortical layer and low expression of neural progenitor-specific genes.[12] Migration of interneurons derived from ventral organoids to the dorsal region was arrested in assembled RTT organoids, indicating the excitatory-inhibitory imbalance, a major phenotype of RTT.[12] However, these previous studies showed only late stages of the RTT phenotype resulting from neuron formation/maturation and neuronal circuit formation in brain development. Reduced expression of neural progenitor markers and increased gliogenesis are also exhibited in RTT organoids.[12,15] Therefore, although the underlying mechanism is not clear, it is well known that MeCP2 plays an important role in the early neurogenesis and gliogenesis of neural progenitor cells (NPCs).

Generation of neural stem cells (NSCs) is spatiotemporally regulated by various signaling pathways in embryonic stem cells (ESCs) during neuroectodermal differentiation.[16–18] Especially, bone morphogenetic proteins (BMPs) are potent morphogens for the patterning, development, and function of the central nervous system.[19] Inhibition of transforming growth factor-beta (TGF-β)/ BMP and WNT pathway directly guides ES cells into dorsal telencephalon by various BMP antagonists and low levels of WNT ligands.[19–21] After neural conversion, NSCs differentiated into NPCs which generate a more restricted repertoire of neurons and glial cells in the ventricular zone of cortical brain.[16] BMP pathway promotes neurogenesis by pushing NPCs toward a neuronal lineage, and Neurogenin-1, the pro-neuronal bHLH transcription factor, is tightly associated with the BMP-dependent cell fate determination.[22–26] Thus, BMP pathway definitely needs to be suppressed for the expansion of NPC before neurogenesis. Indeed, BMP exposure to primary neural stem cells arrested EGF-derived mitosis and promoted TUBB3 expression for neurogenesis.[26] Therefore, precise regulation of BMP pathway according to brain developmental stage is important for the maintenance of NPC pool and the generation of neurons.

Here, we established a new organoid model of RTT brain using MeCP2-truncated mutant iPSCs and confirmed its abnormality of neural rosette formation and NPC proliferation. Total transcriptome analysis of RTT organoids revealed alterations in the BMP signaling pathway, corresponding to elevated levels of phosphorylated SMAD1/5, which are direct regulators of BMP signaling activation. Inhibition of BMP in RTT organoids partially rescued the cell cycle progression of NPCs, which critically exerted the fate commitment of newborn excitatory glutamatergic neurons. In addition, early inhibition of BMP pathway reduced astrocyte maturation which was highly induced by MeCP2 dysfunction. Thus, our results provide insight into a novel function of MeCP2 in NPC expansion and BMP regulation for fate commitment of NPCs to glutamatergic neurons and maturation of early astrocytes.

Materials and Methods

CRISPR/Cas9 gene editing for MeCP2-hTM iPSCs

The MeCP2 indel mutation was induced by using a previously described CRISPR/Cas9 gene editing strategy.[27] Briefly, PX459 vector (62988, Addgene) was cloned with an oligonucleotide targeting MeCP2 exon 4 region. Indels were introduced into IMR90-4 iPS cells (IMR90-4, Wicell Research Institute Inc.) using the Neon Transfection System (Thermo Fisher Scientific, USA). Transfected cells were sorted as a single cell and cultured in the matrigel-coated 96-well plate with mTeSR plus media (100-0276, STEMCELL Technologies) until colony formation. DNA of each clone was harvested and analyzed in the MeCP2 exon region through next-generation sequencing (NGS) and Sanger sequencing. We choose two clones (MeCP2-hTM and hTM2) exhibiting the out of frame due to hTM: 4 bps deletion and 6 bps insertion (ACTTTT) and hTM2: 1 bp deletion in target region of single MeCP2 allele. We used hTM cells as a major RTT iPSCs in this study.

Induced pluripotent stem cells (iPSCs) and cortical organoid culture conditions

Human iPSCs were cultured on the matrigel-coated 6-well cell culture plate with mTeSR Plus media and passaged every 3–4 days by Gentle Cell Dissociation Reagent (100–0485, STEMCELL Technologies) treatment. Cortical organoids were produced as previously described.[28] Briefly, when passage number 3 or 4 of iPSCs, cells were dissociated into single cells by treatment of Accutase (07922, STEMCELL Technologies). A total of 9000 cells were seeded in each well of 96-well ultra-low attachment plate (7007, Corning) and cultured for 10 days in the neural induction media. After then, organoids were transferred to 6-well ultra-low attachment plate (8 organoids/well; 3471, Corning) and cultured for 8 days in the neural differentiation media without vitamin A. From Day 18, organoids were cultured in the neural differentiation media with vitamin A and media were replenished every 4 days.

Detailed methodologies

Details of all reagents and chemicals are provided in Supplementary materials and methods and Table S3. Other detailed methodologies (immunofluorescence, image

analysis, Western blotting, RNA preparation, qPCR analysis, total RNA sequencing, and data statistics) are presented in Supplementary materials and methods.

Results

Loss of MeCP2 function reduces neural rosette formation in the early stage of brain development

The *MECP2* gene is located on the X chromosome, and one of the alleles is randomly inactivated during embryogenesis.[29] Thus, mosaicism occurs as a result of heterozygous *MECP2* mutations, and the ratio to normal varies from patient to patient.[30–33] To overcome phenotypic heterogeneity and investigate the ultimate function of MeCP2 in brain development, human iPSCs (IMR90-4) were genetically engineered using CRISPR/Cas9 system and MeCP2-truncated mutation (clone 1: c.654_657del, 6 bps ins, p.K231Lfs*28 and clone 2: c.655Adel, p.K231Sfs*27) causing complete dysfunction of MeCP2 was introduced (Fig. 1A; Fig. S2A). Although this mutation was occurred heterozygously (MeCP2-heterozygous truncation mutant; MeCP2-hTM), the normal *MECP2* gene was not expressed because it was thought to be on the inactivated X, confirming that this status continued throughout ectodermal differentiation (Fig. 1B,C; Fig. S2B).

To investigate whether MeCP2 loss-of-function affects neural progenitor cells (NPCs) and early brain development, we generated brain organoids using MeCP2 wild type (WT) and hTM iPSCs as previously described.[28] At Day 25, two types of hTM organoids showed significant

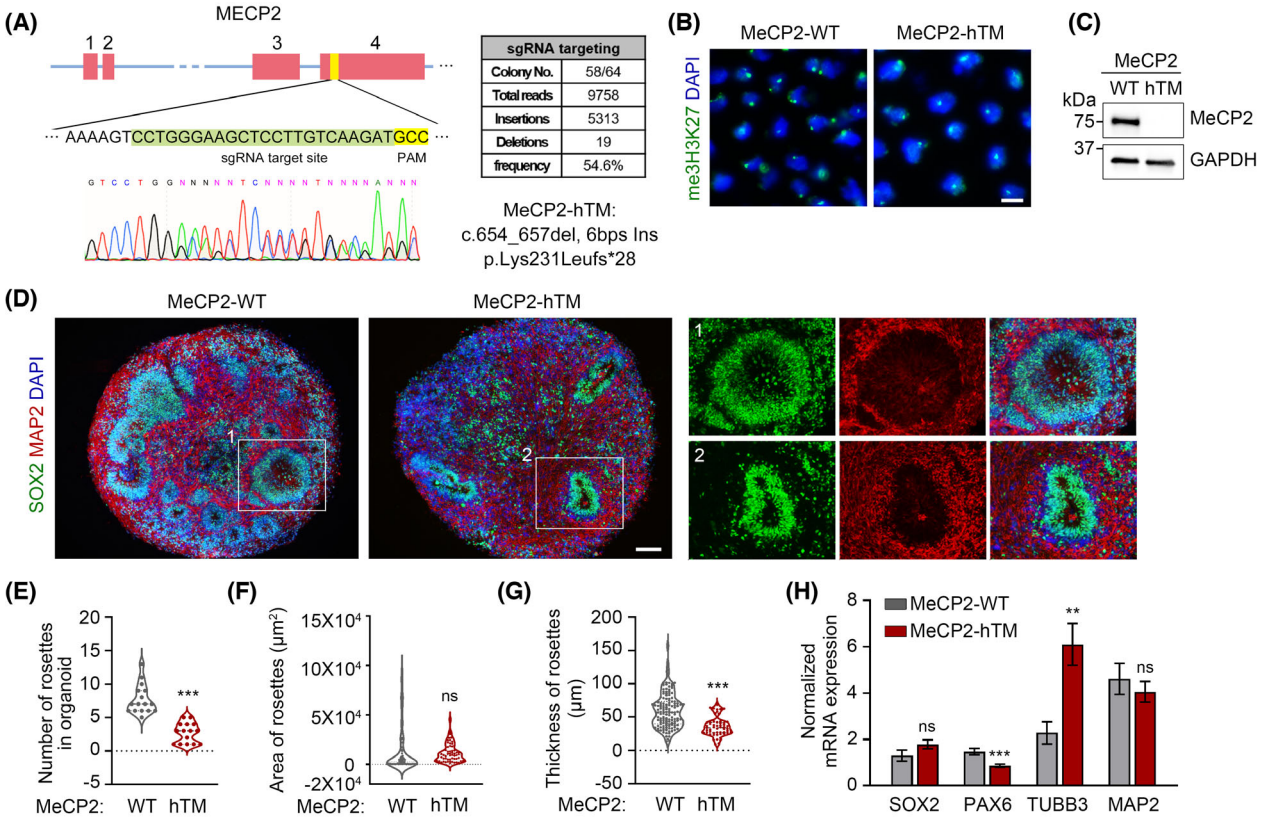

Figure 1. Truncated mutation of MeCP2 causes defect of neural rosette formation. (A) Schematic diagram showing target exon region of MeCP2 and CRSPR/Cas9 gene editing profiles (B) Immunofluorescence images presenting inactivated X chromosome foci labeled by anti-trimethyl-Histone H3 (Lys27) antibodies in MeCP2-WT and -hTM iPSCs. The nucleus was stained with DAPI. (C) Immunoblots showing protein levels of MeCP2 and GAPDH in WT and hTM organoids. GAPDH was a total protein loading control. (D) Immunofluorescence images showing neural rosettes (SOX2 positive) and newborn neurons (MAP2 positive) in WT and hTM organoids at Day 25. Insets present magnified views of indicated rosettes. (E–G) Dot plots presenting the number of rosettes (E), the area of rosettes (F), and the thickness of rosettes (G) in WT and hTM organoids at Day 25. 3 batches, 3–6 organoids were counted in each group. (H) Bar graph showing normalized mRNA expression of indicated genes in WT and hTM organoids at Day 25. 3 batches/3–4 organoids were analyzed in each group. All data are expressed as the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ and ns: not significant. Scale bars: 10 μm (B) and 100 μm (D).

depletion of neural rosettes, the groups of cells which radially arranged surrounding lumen-like structure and expressed NPC markers such as SOX2 and PAX6 (Fig. 1D,E; Figs S1A and S2C–E). Extensive rosette areas marked by SOX2 were well observed in WT organoids, but truncation of MeCP2 reduced the size and thickness of the columnar cell layer in rosettes (Fig. 1F,G; Fig. S2F). Moreover, *PAX6* expression was significantly decreased in hTM organoids at Day 25 (Fig. 1H; Fig. S2G). In addition, the mRNA level of TUBB3, a new neuron marker, was significantly elevated, while MAP2, a more mature neuron marker than TUBB3, did not show significant results in this experiment, but it seems to be lowered. These results indicate that the generation of newborn neurons is transiently induced likely resulting from a lack of PAX6 signaling. Indeed, PAX6 deficiency causes transient production of immature neurons in a conditional mouse model.[34–37] Together, we propose that MeCP2 is required for the formation of neural rosette and maintenance of the NPC pool by mediating PAX6 expression.

MeCP2 dysfunction causes the abnormality of NPC proliferation in VZ-like neural rosettes.

In the early stage of cortical organoids, neural rosettes are composed of polarized neuroepithelial-like cells and resemble the embryonic neural tube.[38] Rosette cells display physiological signs of early NPC and are capable of differentiating into committed neural precursors that generate a series of neurons and glial cells.[38] Thus, precise regulation of NPC expansion is critical to generate the correct number and type of neurons, which depend on both the division mode of NPCs and the length of the cell cycle. PAX6 is a major transcription factor which spatiotemporally controls cell cycle progression of NPCs in early corticogenesis.[35,37] To investigate whether MeCP2-mediated PAX6 depletion affects NPC proliferation, we checked KI67-positive cell populations in neural rosettes from Day 25 organoids (Fig. 2A1; Fig. S2I). Interestingly, the number of KI67-positive NPCs was significantly decreased, and the number of cells expressing Cyclin D1, a transcriptional target of PAX6, was reduced at G1/S transition in hTM organoids (Fig. 2B). These data indicate that MeCP2 dysfunction-mediated PAX6 depletion represses cell cycle progression of NPCs in neural rosettes during early brain development.

The length of cell cycle and the mitotic progression and division orientation of NPCs are tightly regulated for early neurogenesis.[39–41] We assessed mitotic cell populations with their division angle based on the N-Cadherin-positive apical membrane in hTM organoids. It was confirmed that the number of phospho-Histone H3 (PH3)-positive mitotic cells was remarkably reduced (Fig. 2C; Fig. S2J). In addition, the percentage of prophase and prometaphase cells in the entire cell cycle was reduced, but metaphase cells were increased (Fig. 2D). These data indicate that mitotic progression of NPCs is delayed by MeCP2 dysfunction. Moreover, mitotic orientations of NPCs in ventricular zone-like regions were more distorted (Fig. 2D-magnified images and Fig. 2E). Indeed, previous studies have reported that MeCP2 deficiency exerts a tilted angle to spindle poles, and that PAX6 is highly associated with symmetric division of NPCs in early corticogenesis.[37,42,43] Therefore, we suggest that MeCP2 induces PAX6 expression during early brain development and together with PAX6 regulates cell cycle progression of NPCs in the apical region of neural rosettes.

Total transcriptome analysis reveals the alteration of TGF-β/BMP signaling pathway in MeCP2-depleted brain organoid during the early brain development.

Proliferation of NPCs and development of neural rosettes are carefully regulated by the cooperation of various signaling pathways such as Notch, Wnt, and TGF-β/BMP pathways.[38,44–47] To determine which signaling pathways are altered by MeCP2 dysfunction, we analyzed the total transcriptome of type 1 hTM organoid at Day 25. Principal component analysis (PCA) and hierarchical clustering assay revealed differences between WT and hMT organoid samples as well as within-group similarities (Fig. 3A,B). Analysis of differentially expressed genes (DEGs) demonstrated significant changes in the transcriptome of hTM organoids with 2,635 more than twofold upregulated and 1,775 downregulated genes (Fig. 3C; Table S1). These large changes indicate that the role of MeCP2 as a transcriptional modulator is extensive in gene expression. Gene ontology (GO) analysis revealed that genes related to cell fate commitment, anterior–posterior patterning, and hindbrain development were significantly enriched in the upregulated category, whereas genes involved in CNS neuron differentiation, forebrain development, and postsynaptic functions were enriched in the set of downregulated categories (Fig. 3D; Table S2). Intriguingly, we found that 11 of GO terms in cluster 3 and 4 were related to the TGF-β/BMP signaling pathway and that related gene sets were significantly upregulated upon MeCP2 dysfunction (Fig. 3E,F), indicating that MeCP2 is highly correlated with the TGF-β/BMP signaling pathway during early brain development. TGF-β/BMP signaling pathway is tightly involved in the fate determination of early embryogenesis, and inhibition of BMP is essential for the ectodermal differentiation.[48–50] Moreover, activation of

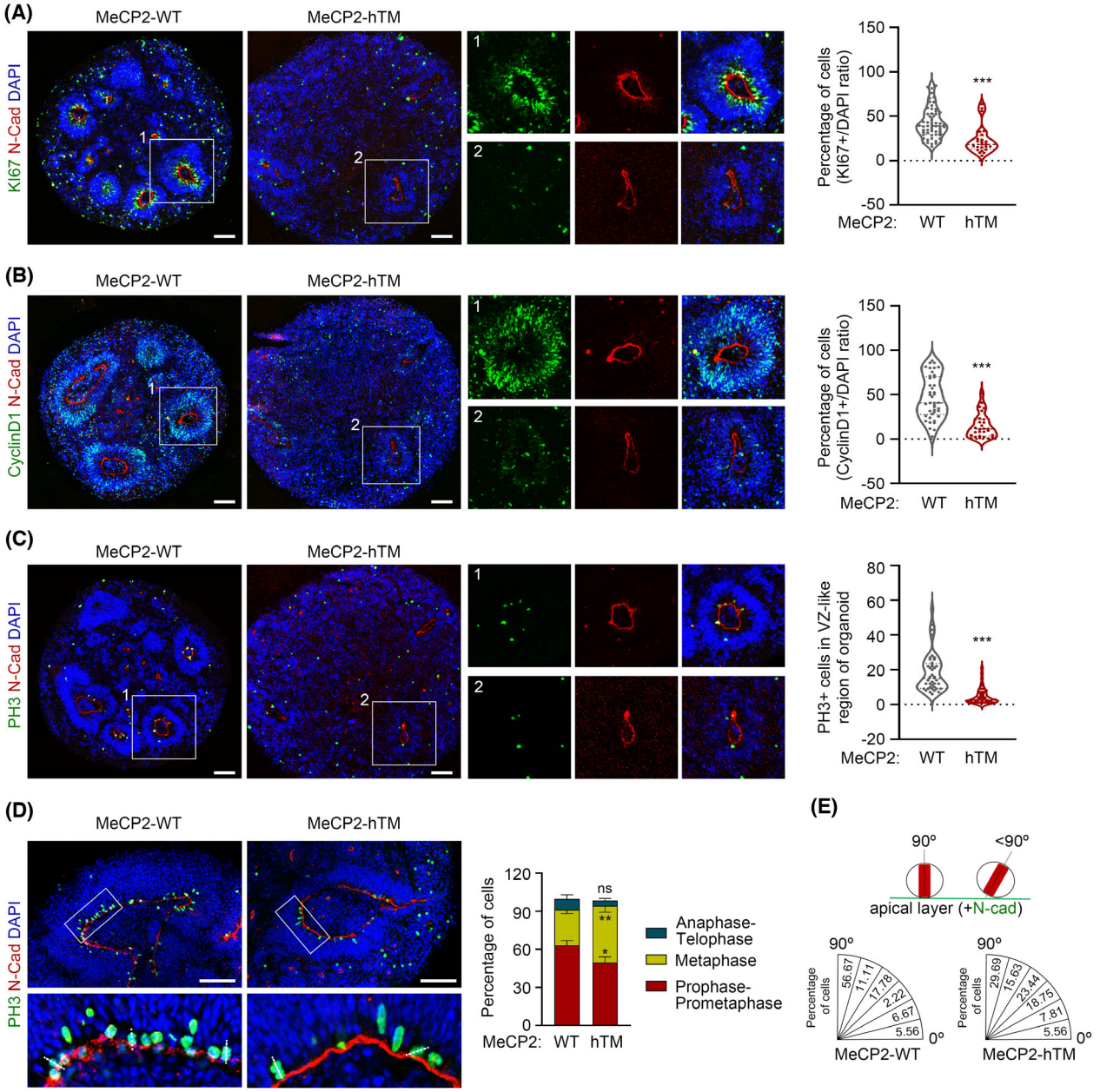

Figure 2. Dysfunction of MeCP2 causes abnormal NPC proliferation in early brain organoids. (A) Immunofluorescence images showing cycling cells in WT and hTM organoids at Day 25. Anti-KI67 antibodies were used for labeling cycling cells in neural rosettes and anti-N-cadherin antibodies marked the apical membrane of lumen-like structure in rosettes. The nucleus was labeled with DAPI. Insets present magnified views of indicated rosettes. The dot plot shows the percentage of KI67-positive cells in rosettes of both groups. (B) Immunofluorescence images showing Cyclin D1-positive cells in WT and hTM organoids at Day 25. Insets present magnified views of indicated rosettes. The dot plot shows the percentage of Cyclin D1-positive cells in rosettes of both groups. (C) Immunofluorescence images showing mitotic cells in WT and hTM organoids at Day 25. Anti-phospho-Histone H3 (PH3) antibodies were used for marking mitotic cells. Insets present magnified views of indicated rosettes. The dot plot presents the number of PH3-positive cells in N-cadherin positive, apical region of rosettes in both groups. (D) Immunofluorescence images showing mitotic cells in rosettes of WT and hTM organoids at Day 25. Insets show magnified views of indicated regions. Dotted lines indicate the axis of division based on apical membrane (N-Cadherin positive). The bar graph shows the percentage of cells in each process of mitosis in both groups. (E) Histograms showing the frequency of mitotic axis angles determined by PH3 and N-Cadherin staining in WT and hTM organoids at Day 25. All data are expressed as the mean ± SEM and 3 batches/3–6 organoids were analyzed in each group. *$p < 0.05$, ***$p < 0.001$ and ns: not significant. Scale bars, 100 μm.

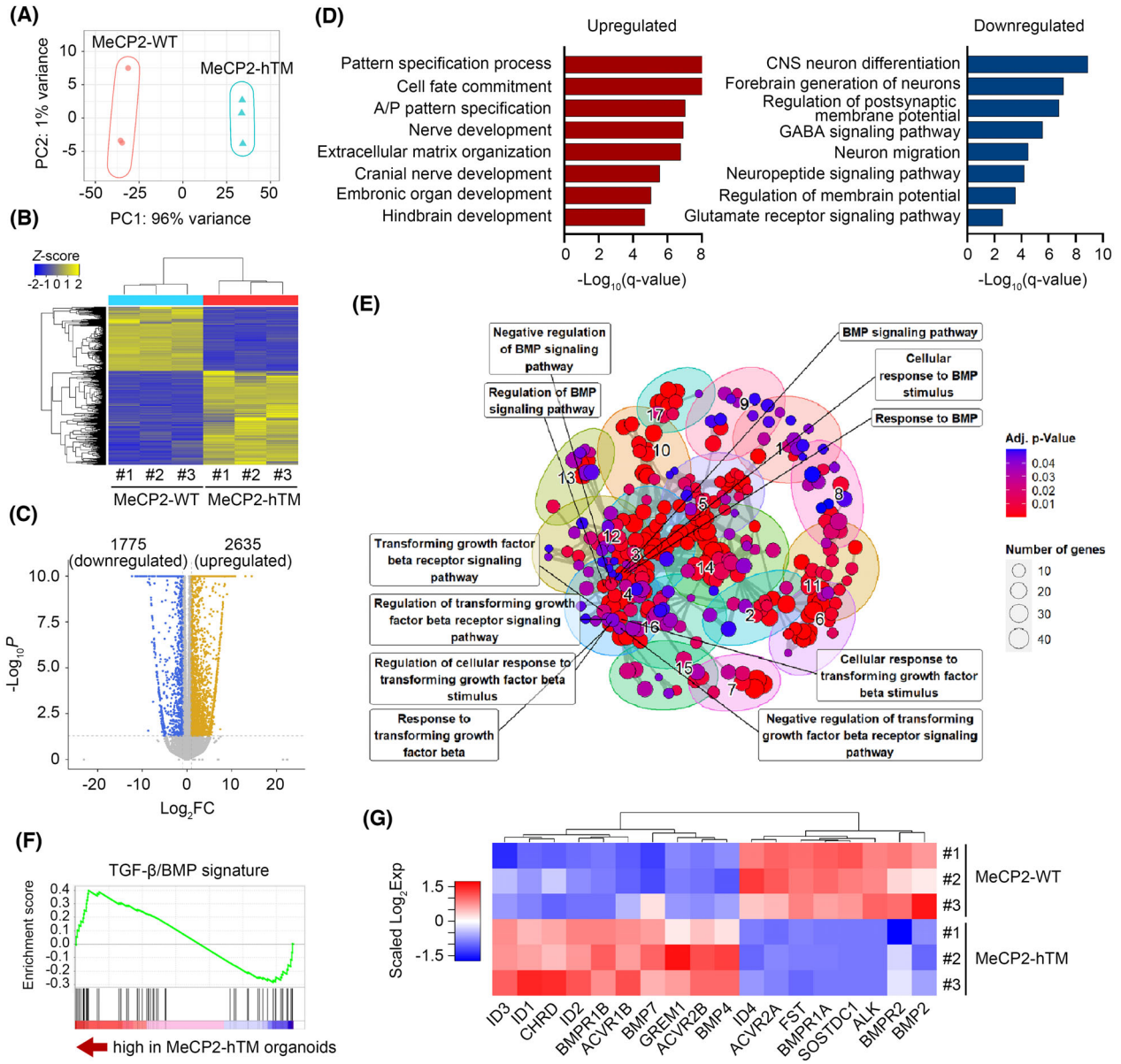

Figure 3. TGF-β/BMP signaling pathways are altered in MeCP2-hTM organoids at Day 25. (A and B) Principal component analysis (PCA) plot (A) and hierarchical clustering heat map (B) presenting the transcriptomic distance and similarity among the WT and hTM organoids samples (n = 3). (C) Volcano plot showing log scaled fold change (FC) and statistical significance (p-value) of each gene calculated through the differentially expressed gene (DEG) analysis between WT and hTM. (D) Bar graphs showing the lists of upregulated or downregulated gene ontology (GO) terms in hTM organoids compared with WT. (E) Dot plot with clusters presenting significant GO terms (dots) and their clusters (colored eclipses) of significant DEGs (adjusted p-value <0.05, |fold change| >2). Size and color of dots mean the number of significant DEGs and statistical significance of GO terms. Color range from red to blue corresponds to the p-value range from 0 to 0.05. (F) Line graph of gene set enrichment analysis (GSEA) showing the correlation between MeCP2 dysfunction and TGF-β/BMP signaling pathways in brain organoid at Day 25 (normalized enrichment score = 1.247, nominal p-value = 0.0889 and FDR (false discovery rate) q-value = 0.841). (G) Heat map showing expression of BMP signaling pathway-related genes in indicated sample groups. Color of bar indicates scaled logarithm based on 2 of normalized expression (Scaled Log_2Exp).

BMP pathway is initiated at the onset of the neurogenesis and suppressed gliogenesis.[22,23,26,51] Therefore, these results indicate that MeCP2 is definitely connected with TGF-β/BMP signaling pathway in neural fate differentiation and neural progenitor expansion in early brain development.

Early activation of BMP pathway caused by MeCP2 depletion lowers cell cycle progression of neural progenitors.

To investigate whether MeCP2 loss-of-function causes excessive activation of TGF-β/BMP pathway, we checked the phosphorylation level of SMAD1/5 and SMAD2/3, key downstream factors of canonical BMP and TGF-β signaling, in hTM organoids at serial time points of neuroectodermal differentiation. Immunofluorescence signals of pSMAD1/5 were high in hTM organoids at Day 25 and this phenomenon was already exhibited at Day 10 when iPSCs just differentiated into the neuroectoderm lineage by treatment of BMP and TGF-β inhibitors (Fig. 4A,B). Moreover, expression of direct BMP target genes such as *ID1*, *ID2*, and *ID3* was significantly upregulated in hTM organoids during early brain development (Fig. 4C; Fig. S2H). However, levels of total and phosphorylated SMAD2/3 were consistently low in hTM, implying the opposing activation between BMP and TGF-β signaling in hTM organoids (Fig. 4B). Indeed, phosphorylation of SMAD1/5 and SMAD2/3 is mutually inhibited and SMAD2/3 double knockout caused high levels of pSMAD1/5 and *ID1* expression, leading to defect of neuroectodermal patterning.[52–54] Thus, these results demonstrate that complete loss of MeCP2 function causes excessive activation of the BMP signaling pathway during neuroectodermal differentiation of brain organoids.

A previous study reported that, unlike other regions of CNS, such as the spinal cord, exposure of BMP ligands to mouse forebrain in the early developmental stage significantly suppressed cell proliferation and induced cell death.[55] These results demonstrated that BMPs negatively regulate cell proliferation and survival in the early forebrain. In this context, to investigate whether defects of rosette formation and cell proliferation are derived from the excessive activation of BMP pathway, we treated high doses of LDN193189 (LDN) or Dorsomorphin (DM) during the first 10 days of neural induction (Fig. 4D). LDN and DM are specific inhibitors of activin-receptor like kinase (ALK) 2/3/6 which directly phosphorylated SMAD1 and 5 for switching on the canonical BMP pathway. Indeed, in a widely used brain organoid differentiation protocol, 0.1 μM of LDN was routinely treated with 10 μM of SB431542, an inhibitor of ALK4/5/7 receptors leading suppression of SMAD2/3 phosphorylation, at the period of neuroectodermal differentiation.[28] Since hTM exhibited high levels of pSMAD1/5, we increased the doses of LDN (0.5 μM) and DM (10 μM), but did not change the dose of SB431542 because of the low levels of total and phosphorylated SMAD2/3. At Day 10, the protein levels of pSMAD1/5, the BMP target genes, were significantly reduced in both groups, but these effects were only persisted in the LDN group until Day 25 (Fig. 4E). Moreover, LDN or DM treatment effectively reduced expression of BMP target genes during 10 days, but the inhibitory effect gradually disappeared after drug treatment was stopped (Fig. 4F). Total and phosphorylated SMAD2/3 levels in LDN group increased slightly more than in the DM.

At Day 25, we found that the number of rosettes and KI67-positive cells was increased by BMP inhibition in hTM organoids (Figs S1B,C and S2I; Fig. 4G). Moreover, levels of Cyclin D1 were elevated in LDN group compared with not treated (NT) MeCP2-hTM organoids (Fig. S1E). However, size of rosettes and mitotic cells in apical region (N-Cadherin positive) of rosettes were not rescued by BMP inhibition (Fig. S1D and Fig. S2L,J; Fig. 4G). These results indicate that BMP inhibition in hTM organoids slightly rescues the cell cycle progression of NPCs, but does not correct mitosis regulation, leading to the formation of small-sized rosettes. We further confirmed that PAX6 expression was still low in both groups until Day 25 (Fig. S1F). Previous report demonstrated that when the embryonic stem cells differentiate into neuroectodermal progenitors, the level of PAX6 transcripts gradually increases, which promotes miR-135b expression for efficient inhibition of BMP pathway.[56] Another study showed that miR-135b was negatively regulated by DNA methylation and MeCP2 correlated with miR-135b suppression.[57] Thus, elevated activation of BMP pathway appears to be due to MeCP2 dysfunction-mediated PAX6 and miR-135b depletion, and inhibition of BMP pathway slightly overcomes the phenotype of PAX6 depletion in hTM organoids during early brain development. Together, we suggest that MeCP2 regulates the precise progression of NPC expansion by modulating PAX6 expression and BMP pathway.

Inhibition of the elevated BMP pathway during neuroectoderm development in MeCP2-depleted brain organoids leads NPCs to commit to becoming glutamatergic neurons.

NPCs in the cerebral cortex typically generate the pyramidal projection neuron which migrates radially to the cortical plate and expresses excitatory neuronal markers such as VGLUT1 during the neurogenesis.[58–60] Most studies using cultured dorsal forebrain organoids have shown that glutamatergic neurons are primarily generated during neurogenesis, as in the human embryonic cortex.[61] However, inhibitory interneurons (INs) are spontaneously generated in these organoids, and they are distinct from INs derived from the NKX2.1-positive ventral forebrain.[61] We checked that glutamatergic and GABAergic neurons were

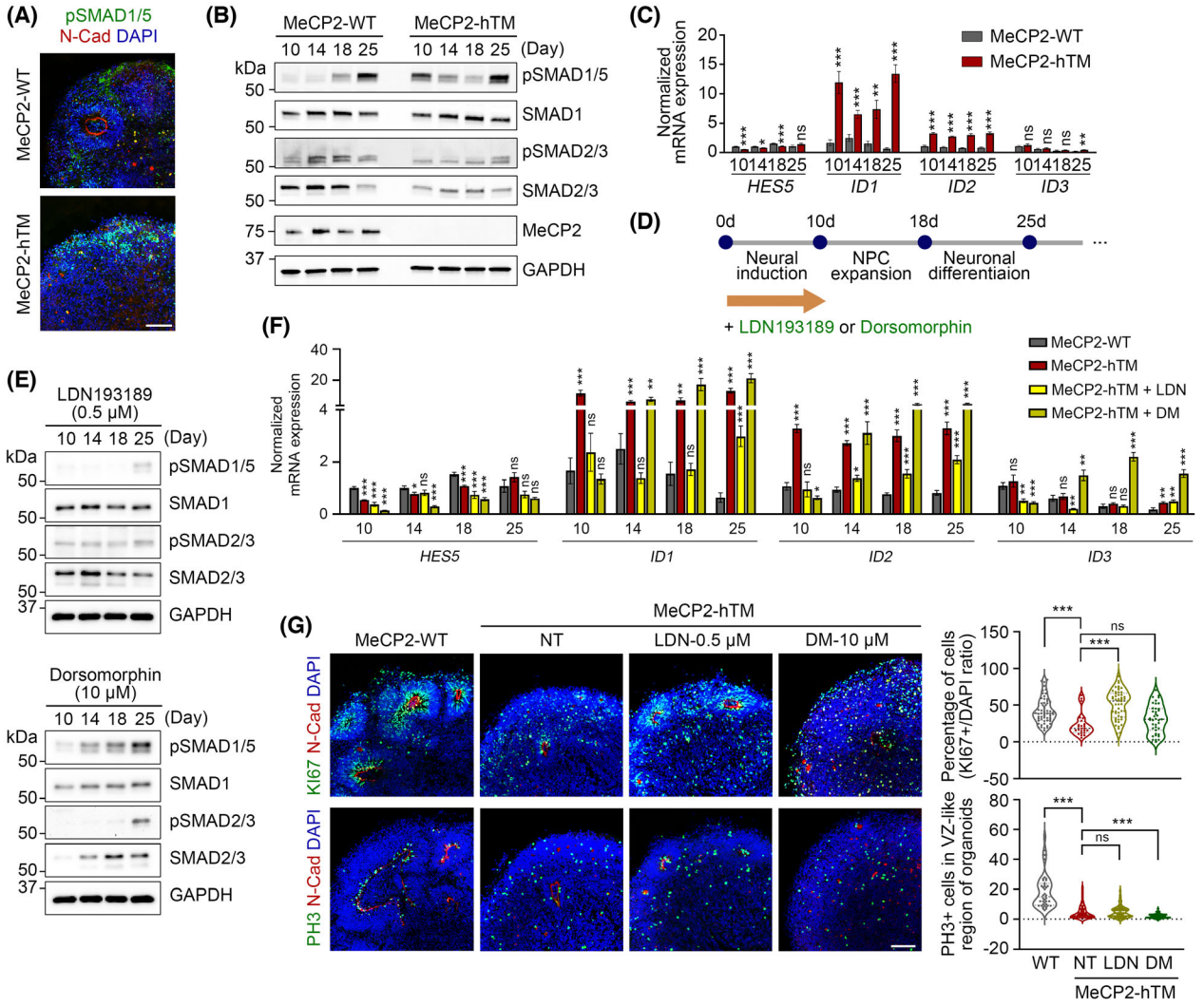

Figure 4. Inhibition of BMP pathway slightly rescues the cell cycle of NPC in MeCP2-depleted organoids. (A) Immunofluorescence images showing phosphorylated SMAD1/5-positive cells in WT and hTM organoids at Day 25. (B) Immunoblots presenting protein levels of pSMAD1/5, SMAD1, pSMAD2/3, SMAD2/3, MeCP2, and GAPDH in indicated development time points of WT and hTM organoids. GAPDH was a total protein loading control. (C) Bar graph showing normalized mRNA expression of BMP target genes in indicated time points of WT and hTM organoids. (D) Schematic diagram showing culture methods of organoids and timeline of BMP inhibitor treatment. LDN193189 (LDN) and dorsomorphin (DM) were treated 0.5 μM and 10 μM on other days for 10 days, respectively. (E) Immunoblots showing protein levels of pSMAD1/5, SMAD1, pSMAD2/3, SMAD2/3, and GAPDH in indicated conditions of WT and hTM organoids. (F) Bar graph presenting normalized mRNA expression of BMP target genes in indicated conditions of WT and hTM organoids. (G) Immunofluorescence images showing KI67-positive cycling cells and PH3-positive mitotic cells in indicated condition of WT and hTM organoids at Day 25. Dot plots show the percentage of KI67-positive cells and the number of PH3-positive cells of rosettes in indicated conditions of WT and hTM organoids (KI67-positive cell plot: $p = 0.0200$ in unpaired t-test of LDN vs WT, $p = 0.0862$ in unpaired t-test of DM vs WT; PH3-positive cell plot: $p < 0.0001$ in both unpaired t-tests of LDN vs WT and DM vs WT). NT means "not treated." Quantitative PCR data are expressed as the mean ± SEM and 2 batches/3–4 organoids were analyzed in each group. Quantitative data from immunofluorescence images are expressed as the mean ± SEM and 3 batches/3–6 organoids were analyzed in each group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and ns: not significant. Scale bars, 100 μm.

well generated as neurogenesis progressed in our organoid culture system (Fig. 5A). In particular, the subcellular localization of VGLUT1 gradually became dot-shaped, indicating maturation of excitatory neurons and synapses. On the contrary, loss of MeCP2 function caused the alteration of neuronal fate specification in both excitatory and inhibitory types of neurons compared with MeCP2-WT organoids (Fig. 5A,C,D). These results indicate that MeCP2 dysfunction disrupts maturation of newborn neurons into excitatory or inhibitory categories.

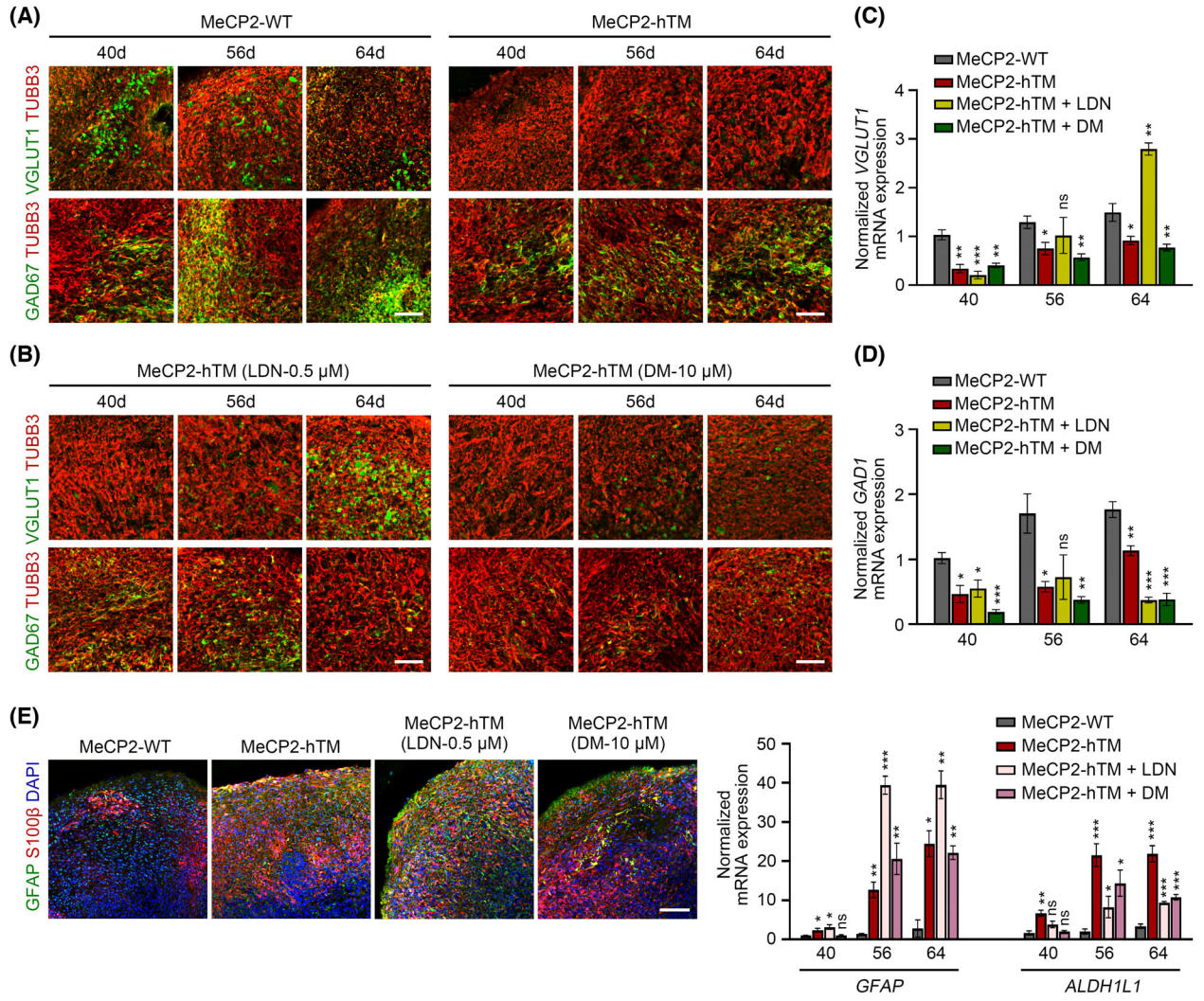

Figure 5. Inhibition of BMP pathway during neuroectoderm development is involved in the glutamatergic neurogenesis and astrocyte specification. (A and B) Immunofluorescence images showing glutamatergic and GABAergic neurons in indicated conditions of WT and hTM organoids at different time points. Anti-VGLUT1 and anti-GAD67 antibodies were used for labeling glutamatergic and GABAergic neurons, respectively. Anti-TUBB3 antibodies were used for labeling whole neurons in organoids. (C and D) Bar graphs presenting normalized *VGLUT1* and *GAD1* mRNA expression in indicated conditions of WT and hTM organoids at different time points. *GAD1* gene was encoded protein GAD67. (E) Immunofluorescence images showing GFAP and S100β positive astrocytes in indicated conditions of WT and hTM organoids at Day 64. The bar graph shows normalized *GFAP* and *ALDH1L1* mRNA expression in indicated conditions of WT and hTM organoids at different time points. All data are expressed as the mean ± SEM and 2 batches/3–4 organoids were analyzed in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ns: not significant. Scale bars, 50 μm (A and B) and 100 μm (E).

Intriguingly, early inhibition of BMP pathway by LDN increased *VGLUT1* mRNA expression, a specific marker of glutamatergic neurons, compared with hTM and even WT organoids (Fig. 5B,C). DM did not rescue the *VGLUT1* expression, predictably. Meanwhile, levels of GAD67 (*GAD1*), a marker of GABAergic neuron, was significantly reduced in LDN and DM groups, indicate that BMP inhibition in neuroectodermal progenitors encourages NPCs to generate glutamatergic neurons when neurogenesis (Fig. 5B,D).

Switching from neurogenesis to gliogenesis differentiates NPCs into glial cells such as astrocytes which maintain neuronal function and provide neurotransmitters to neurons.[62–64] The balance between neuron and astrocyte formation is really important for synaptogenesis and neural circuit formation.[65–67] Dysfunction of MeCP2 increased astrogenesis in conditional RTT mouse models and patient-derived brain organoids, concomitantly with activated BMP signaling pathway.[15] We also confirmed

that specific astrocyte markers, GFAP, S100β, and ALDH1L1 were drastically upregulated in hTM organoids from Day 56 (Fig. 5E). Interestingly, LDN-treated organoids exhibited high levels of *GFAP* expression, but *ALDH1L1* expression was lower than hTM organoids (Fig. 5E; right bar graph). Likewise, DM-treated organoids showed reduced levels of *ALDH1L1* expression, despite the comparable levels of *GFAP* expression to hTM. GFAP is broadly expressed in astroglial lineage cells, but S100β and ALDH1L1 are more highly expressed as astrogenesis progression.[68–70] These results indicate that early inhibition of BMP pathway by LDN treatment induces gliogenesis via GFAP expression, but reduces astrocyte specification in hTM organoids.

Conclusively, our results demonstrate that efficient inhibition of BMP pathway during neuroectoderm development upregulates glutamatergic neurogenesis which is altered by complete loss of MeCP2 function. Although this inhibition of the BMP pathway does not suppress the excessive gliogenesis in MeCP2-hTM organoids, it negatively exerts astrocyte maturation. Therefore, we suggest that regulation of neural fate determination and astrogenesis is linked to MeCP2-dependent BMP signaling in NPCs during early neural development.

Discussion

Developmental disorders such as RTT are usually caused by functional mutations in the causative gene, and these dysfunctional disease models allow the etiological research and treatment strategies to be established.[71–74] In this paper, we genetically engineered exon regions of MeCP2 in iPSCs and developed a new RTT brain organoid model exhibiting complete dysfunction of MeCP2 with unreported defects in the neural rosette formation and neuronal specification related to the BMP signaling pathway.

Previous RTT models with missense mutations in MeCP2 rarely exhibited abnormalities in the neuroectodermal differentiation and NPC expansion.[75–77] This means that point mutations in the functional domain of MeCP2 have relatively less impact on the early events in brain development than functional knockouts. Patients with nonsense mutations in MeCP2 showed severe phenotypes such as microcephaly and even neonatal encephalopathy with short lifespan, as well as classical RTT phenotypes.[78,79] These severities are usually overlapped with symptoms of rare diseases which are caused by NPC abnormalities or neuronal malformations.[80–83] Likewise, MeCP2-trancated mutant RTT organoids we constructed showed early developmental defects in which the BMP signaling pathway was poorly suppressed. This indicates that MeCP2 is also a key regulator of neuroectodermal differentiation and NPC expansion in early brain development.

Intriguingly, a recent study demonstrates that MeCP2-null brain organoids exhibited defects of radial glial cells in neural rosettes at Day 60, but did not show NPC abnormalities at Day 30.[84] However, it should be noted that these organoids were cultured with matrigel, an extracellular matrix (ECM)-based hydrogel capable of providing ECM signals to organoids for precise development of brain organoids.[85] It has been well-known that laminin, a major component of matrigel, significantly promotes the NPC proliferation, as well as neurogenesis and neurite outgrowth.[86–88] In addition, collagen type IV, another component of matrigel, has been reported to promote NPC proliferation and neural differentiation.[89,90] Interestingly, extracellular matrix organization (GO: 0030198)-related genes which are involved in the disassembly of ECM were upregulated in our RTT organoids cultured in suspension without matrigel (Fig. 3D; Table S2), indicating the possibility of correlation between MeCP2 and ECM production. Thus, we speculate that additional ECM signals can partly compensate for MeCP2 dysfunction in the early phase of brain organoids.

During neural conversion of pluripotent stem cells, PAX6 expression is gradually increased for NPC pool- and telencephalon-specific fate determination.[91–93] PAX6 is a key transcription factor that directly regulates NPC proliferation and symmetrical division angle to sustain self-renewal of NPCs before the generation of neurons.[34,35,37] However, dysfunction of MeCP2 caused significant depletion of PAX6 and its direct target, Cyclin D1 in NPCs of neural rosettes at Day 25. Moreover, KI67-positive cycling cells and PH3-positive mitotic cells were remarkably reduced in MeCP2-depleted organoids. A previous study also reported that levels of Cyclin D1 and BrdU incorporation rates were reduced in MeCP2-null mouse NPCs, indicating suppression of S phase entry.[94] Thus, we suggest that MeCP2 is required for PAX6 expression and is involved in the cell cycle progression of NPCs during early brain development.

Through the total transcriptome analysis, we confirmed that BMP signaling pathways were significantly altered in MeCP2-hTM organoids at Day 25, and this abnormality was already shown on Day 10 with high levels of pSMAD1/5. Inhibition of BMP pathway is critical for the fate conversion of stem cells into neuroectoderm lineage by triggering expression of key transcription factors such as PAX6.[50,51,55,56,92,93] PAX6 further reinforces BMP inhibition through miR135b-dependent translational regulation, which accelerates neural induction and niche formation for NPCs[53]. Intriguingly, a recent report suggested the negative correlation between MeCP2 and miR135b expression.[57] In this paper, we showed that forebrain development and glutamate receptor signaling pathway-related genes were notably affected by MeCP2

dysfunction in gene ontology analysis. Therefore, we suggest that MeCP2 is tightly associated with the suppression of BMP activation and PAX6 expression during neuroectodermal differentiation, implying that MeCP2 plays a role in the brain region specification and, critically, in the telencephalon development.

High-dose LDN treatment mitigated MeCP2-hTM-mediated BMP hyperactivation, slightly rescued rosette formation and increased cycling cell population, but did not induce PAX6 expression. This means that BMP inhibition is insufficient to overcome the defect of transcriptional repertoire associated with early forebrain development in hTM organoids. Indeed, neuroectodermal differentiation is a highly cooperative process with other signaling pathways such as FGF and WNT, and the involvement of these signals is sufficient to promote neural conversion regardless of BMP inhibition.[95–98] Moreover, PAX6 expression is systematically regulated by binding of transcription factors at its enhancer regions, which is modulated by involvement of FGF and WNT signaling pathways.[99–102] In particular, FGF- and WNT-related genes were also affected by MeCP2 depletion in total transcriptome analysis, implying that MeCP2 is broadly involved in the regulation of signaling pathways for fine-tuned progression of brain development (Table S2). Therefore, we suggest that phenotypes of MeCP2 dysfunction in RTT organoids are consequences of extensive alteration of signaling pathways directly or indirectly affected by MeCP2-mediated transcriptional regulation.

Although BMP inhibition did not fully rescue dysfunction of MeCP2, we found that early inhibition of the BMP pathway led MeCP2-depleted NPCs to generate glutamatergic neurons during neurogenesis. Glutamatergic fate specification is spatiotemporally regulated by serial expression of transcription factors such as NGN2, NEUROD, and TBR1 in NPCs and newborn neurons of developing forebrain.[103–106] MeCP2 is required for the glutamatergic neurogenesis and controls excitatory synaptogenesis for learning and cognitive ability of brain.[12,107–109] We also found that loss of MeCP2 function significantly reduced VGLUT1 expression and excitatory synapse maturation. However, pharmacological inhibition of BMP pathway during neural conversion of hTM iPSCs changed the fate commitment of MeCP2-depleted NPCs into excitatory glutamatergic neurons. This indicates that amelioration of the altered BMP pathway in neuroectodermal development of RTT organoids can repair the trajectory of neural fate which is mired by MeCP2 dysfunction. It is noted that GABAergic neurons spontaneously generated in the organoid system were also altered by MeCP2 loss, but early inhibition of BMP pathway drastically exacerbated this inhibitory neurogenesis. Dorsal forebrain consists mainly of excitatory neurons, but inhibitory interneurons originate from ventral telencephalon and migrate to dorsal region.[110–113] Therefore, our results demonstrated that early inhibition of altered BMP pathway specifically guides the fate of MeCP2-depleted organoids to excitatory neurogenesis.

Unlike neurogenesis, gliogenesis is aberrantly induced in RTT patients and various models.[114,115] Levels of GFAP, a predominant marker of astrocytes, are significantly increased in RTT patient,s and MeCP2 represses the expression of GFAP by directly binding to its methylated promoter region.[115,116] Our RTT organoids also exhibited excessive expression of astrocyte markers such as GFAP, S100β, and ALDH1L1 at Day 64. Intriguingly, LDN-mediated BMP inhibition during neuroectoderm development intensively increased GFAP expression in RTT organoids, but decreased ALDH1L1. These results indicate that overactivation of BMP pathway in neural conversion of MeCP2-hTM iPSCs drastically induces gliogenesis but reduces astrogeneitic maturation via suppression of astrocyte-specific marker expression. In this paper, we did not reveal the detailed molecular mechanisms underlying these phenomena, but we suggest a link between MeCP2-mediated BMP regulation of neuroectodermal cells and astrocyte generation. Future studies are needed to dissect the significance of MeCP2 roles in astrocytic fate specification, relating to BMP pathway regulated in neuroectoderm development.

Together, we propose a novel function of MeCP2 in neural conversion and NPC expansion that may exert neural fate specification and astrogenesis in late periods of brain development. Our findings contribute to understanding the roles of MeCP2 in brain development and provide clues about the pathogenic mechanisms of RTT that have been difficult to explain in existing models.

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

HH and HC designed of this study and wrote this manuscript. HH performed most of the experiments and analyzed the data. SBY, JEP, and JIL contributed to the acquisition and analysis of the data. HYK and HJN contributed to the establishment and maintenance of MeCP2-hTM iPS cells. HC supervised the whole project. All authors have confirmed and approved the final version.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1.
Figure S2.
Table S1.
Table S2.
Table S3.
Appendix S1.
Captions.

